THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

III. Physiological Significance. The Influence of Trypsin and of Antiprotease on Bacterial Growth and Sulfonamide Action

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Sir A. E. Wright reported in 1916 that the addition of trypsin, leucocytes, or leucoprotease to serum permitted the growth of bacteria which would otherwise be unable to grow in serum. His explanation for this finding was that the trypsin inhibited the antiproteolytic activity of the serum and thereby permitted the accumulation of products of protein digestion, which he believed were necessary for the growth of certain bacteria (called by him "serosaprophytes").

Teale has more recently (1933) disputed this explanation, reporting experiments to show that the presence of antiprotease in the serum does not influence bacterial growth and pathogenicity. He has also offered evidence against the theory of Jobling and Petersen (1914) that the toxins produced during bacterial infection, those responsible for anaphylactic shock, and those present in "anaphylotoxin," are all of the same nature and due to the same cause: namely, to the absorption of antiprotease from the serum, and the consequent degradation of protein substances by the uninhibited action of proteolytic enzymes. However, he reported the interesting observation that although an infected animal may die with the antiproteolytic activity of its serum high, low, or unchanged, animals dying rapidly usually possessed low antiproteolytic activity, while those dying slowly usually showed high activity.

The presence of a powerful antiprotease in the blood and in inflammatory exudates, and the presence of considerable leucoprotease in most foci of infection, render important exact information concerning the effect of protease and of antiprotease on the growth of pathogenic bacteria. For this reason the growth of several organisms was followed in serum with (1) antiprotease intact, (2) additional antiprotease added, (3) antiprotease destroyed, and (4) trypsin added; and in peptone, meat infusion, and albumen solution, with and without added trypsin. And in order to investigate the effect of protease and of antiprotease on bacterial growth when inhibited by a sulfonamide drug, the growth of organisms in the above media was also followed in the presence of sulfathiazole (at 10 mg. per cent concentration).

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The growth of *Escherichia coli*, *Eberthela typhi*, *Proteus vulgaris*, *Staphylococcus aureus*, and *Streptococcus pyogenes* was followed in the following media:

I. Serum with antiprotease intact.

1. 12.5 per cent rabbit's serum (heated at 56°C. for 30 minutes to destroy complement, so as to eliminate the action of normal bactericidal antibodies).
2. 1 + trypsin (0.5 mg./cc. sterile crude trypsin).
3. 1 + sulfathiazole (0.1 mg./cc.).
4. 1 + sulfathiazole (0.1 mg./cc.) + trypsin (0.5 mg./cc.).
5. 1 + pancreatic trypsin inhibitor (2.5 mg./cc.).

(In the following media concentrations are as in I, except where otherwise indicated.)

II. Serum with antiprotease destroyed.

1. 12.5 per cent rabbit's serum (heated at 80°C. for 10 minutes. This completely destroys antiproteolytic activity (Fujimoto, 1918, and by test)).
2. Serum + trypsin.
3. Serum + sulfathiazole.
4. Serum + sulfathiazole + trypsin.

III. Peptone.

1. 1 per cent peptone (nutri-peptone,[1] Wilson) (containing 0.5 per cent NaCl and adjusted to pH 7.4).
2. 1 per cent peptone + trypsin.
3. 1 per cent peptone + sulfathiazole.
4. 1 per cent peptone + sulfathiazole + trypsin.

IV. Meat infusion.

1. 2 per cent beef heart (Difco) (containing 0.2 per cent Na_2HPO_4 and 0.36 per cent NaCl and adjusted to pH 7.4).
2. 2 per cent beef heart + trypsin.
3. 2 per cent beef heart + sulfathiazole.
4. 2 per cent beef heart + sulfathiazole + trypsin.

V. Albumen.

1. 1 per cent crystalline egg albumen (containing 0.2 per cent Na_2HPO_4 and 0.36 per cent NaCl and adjusted to pH 7.4).
2. 1 per cent crystalline egg albumen + trypsin.
3. 1 per cent crystalline egg albumen + sulfathiazole.
4. 1 per cent crystalline egg albumen + sulfathiazole + trypsin.

The growth of the bacteria in these media was followed by measuring the turbidity of the bacterial suspensions at various intervals by means of a photoelectric cell. The opacity of a bacterial suspension of any age is represented as the number of 24 hour old organisms which, when suspended in the same medium, allow the same amount of light to be transmitted. The number of these organisms was determined by direct count of stained bacteria, and a control curve for each organism and each medium was drawn which allowed translation

[1]A mixture of thio-peptone (tryptic "digest" of lung) and medo-peptone (tryptic "digest" of casein).

of turbidity measurements into corresponding numbers of 24 hour old organisms. Obviously this translation is not an exact one, since as growth proceeds the size, refractivity, and reflectivity of the bacteria vary in addition to their number. However, the work of Wilson (1926) and of others indicates that the opacity measurement is probably an accurate measure of the total protoplasmic mass. And this is a truer measure of growth than bacterial number. Such possible sources of error as change in the color or opacity of the medium as a result of bacterial action, or merely as a result of incubation, were proved negligible by filtering off the bacteria from control suspensions after varying incubation periods, and determining the opacity of the medium.

The initial concentration of bacteria in each case was approximately 5 million/cc. The organisms were obtained from 5 day old agar slant cultures (to assure appreciable bacteriostatic activity by the sulfathiazole, which, like the other sulfonamide drugs, acts best on older organisms), and were washed in saline before addition to the media.

Examination of the data in Table I and Charts A to E reveals the following:

1. Growth proceeded more rapidly and further in serum that had been heated at 80°C. for 10 minntes than in serum that had merely been heated sufficiently to destroy complemeut.

It cannot be concluded with certainty that this difference is due even in part to the destruction of serum antiprotease, but it seems very likely. It has been repeatedly demonstrated (*e.g.*, Banting and Gairns, 1930) that trypsin acts much more readily on the proteins of serum heated sufficiently to destroy its antiproteolytic activity than on the proteins of serum with intact antiprotease. Since bacterial proteases are also inhibited by serum (von Dungern, 1898; Kämmerer,1911), they too would be expected to digest serum proteins more readily in the absence of serum antiprotease than in its presence.

2. Growth proceeded more rapidly and further in all media to which trypsin had been added. The increase was greater in the presence of sulfathiazole than in its absence and was most marked in the case of Proteus.

Bainbridge (1911) has found that bacteria (with a few exceptions)are unable to readily digest proteins and perhaps even albumoses and peptones, except when very abundant growth is occurring. He has suggested that they are capable of absorbing only amino acids and lower peptides, and that they can produce proteases only when these protein breakdown products are readily available. This indicates the importance to bacterial growth of the presence of proteases other than bacterial proteases, especially when the concentration of non-protein nitrogen is low compared to that of protein, as in the blood and in exudates.

The data reported above show that the growth-promoting action of trypsin is also exerted (although to a lesser extent) in peptone and meat infusion, in which the concentration of protein is very low. Since crude trypsin, which

TABLE I

The Influence of Trypsin and of Antiprotease on Bacterial Growth and Sulfonamide Action

	Incubation, *hrs.*	*E. coli* millions/cc.					*E. typhi* millions/cc.					*Proteus vulgaris* millions/cc.				
		0	10	20	42	64	0	10	20	42	64	0	10	20	42	64
	Medium															
I	1. Serum	5	130	175	170	170	5	50	140	120	115	5	115	225	225	205
	2. Serum + trypsin	5	140	220	210	185	5	75	275	230	230	5	255	540	540	495
	3. Serum + sulfathiazole	5	50	70	50	10	5	5	25	5	5	5	50	50	50	5
	4. Serum + sulfathiazole + trypsin	5	50	90	95	5	5	45	150	185	165	5	140	185	120	115
	5. Serum + trypsin inhibitor	5	120	130	125	110	5	25	105	100	90	5	70	110	105	105
II	1. Serum heated at 80°	5	310	395	310	290	5	165	160	140	120	5	255	280	210	190
	2. Serum + trypsin	5	210	415	525	345	5	280	410	420	350	5	520	725	565	500
	3. Serum + sulfathiazole	5	105	125	45	45	5	30	50	30	30	5	115	115	135	160
	4. Serum + sulfathiazole + trypsin	5	155	445	385	265	5	95	110	120	130	5	270	630	540	495
III	1. Peptone	(Omitted)					5	45	245	245	240	5	180	260	255	250
	2. Peptone + trypsin						5	50	340	300	240	5	445	560	560	560
	3. Peptone + sulfathiazole						5	35	95	90	75	5	30	280	280	260
	4. Peptone + sulfathiazole + trypsin						5	75	230	235	205	5	45	345	370	285
IV	1. Meat infusion	(Omitted)					5	70	160	115	90	5	140	210	210	210
	2. Meat infusion + trypsin						5	165	330	330	300	5	335	620	625	650
	3. Meat infusion + sulfathiazole						5	85	165	165	120	5	45	195	130	90
	4. Meat infusion + sulfathiazole + trypsin						5	95	180	180	150	5	45	225	375	330
V	1. Albumen	(Omitted)					5	50	95	75	30	5	95	185	140	120
	2. Albumen + trypsin						5	95	190	205	200	5	205	420	460	440
	3. Albumen + sulfathiazole						5	25	25	<1	<1	5	75	25	<1	<1
	4. Albumen + sulfathiazole + trypsin						5	45	45	45	<1	5	95	145	185	165

	Incubation, *hrs.*	*Staphyloccus aureus* millions/cc.					*Streptococcus pyogenes* millions/cc.				
		0	10	20	42	64	0	10	20	42	64
	Medium										
I	1. Serum	5	30	120	140	120	5	50	90	115	105
	2. Serum + trypsin	5	30	190	370	360	5	185	165	165	120
	3. Serum + sulfathiazole	5	70	25	5	<1	5	5	5	5	<1
	4. Serum + sulfathiazole + trypsin	5	75	120	120	50	5	50	165	165	140
	5. Serum + trypsin inhibitor	5	30	110	130	120	5	20	45	55	55
II	1. Serum heated at 80°	5	255	250	235	235	5	140	165	165	160
	2. Serum + trypsin	5	455	435	435	345	5	355	355	400	400
	3. Serum + sulfathiazole	5	95	25	25	5	5	25	25	<1	<1
	4. Serum + sulfathiazole + trypsin	5	450	450	380	335	5	140	165	170	170
III	1. Peptone	5	380	380	420	420	5	90	130	130	110
	2. Peptone + trypsin	5	465	485	555	555	5	145	210	220	200
	3. Peptone + sulfathiazole	5	140	250	250	220	5	45	75	75	25
	4. Peptone + sulfathiazole + trypsin	5	320	390	450	450	5	140	95	75	75
IV	1. Meat infusion	5	275	300	380	340	5	100	110	100	95
	2. Meat infusion + trypsin	5	465	465	520	540	5	250	245	240	220
	3. Meat infusion + sulfathiazole	5	230	230	240	245	5	70	45	45	45
	4. Meat infusion + sulfathiazole + trypsin	5	275	375	400	375	5	110	200	205	235
V	1. Albumen	5	95	25	25	<1	5	70	75	85	80
	2. Albumen + trypsin	5	135	225	250	275	5	100	125	120	120
	3. Albumen + sulfathiazole	5	75	5	5	<1	5	25	5	<1	<1
	4. Albumen + sulfathiazole + trypsin	5	120	140	150	170	5	50	80	100	100

was used, contains both protease and peptidase, growth acceleration as a result of peptidase action is indicated in addition to the protease action.

Since leucoprotease has tryptic (protease) action its growth-promoting properties (especially when the N. P. N. in the medium is relatively low) must be considered. And since serum antiprotease exerts its buffering action against leucoprotease, just as it does against trypsin (Opie, 1905, repeatedly confirmed) it too is very probably important with respect to bacterial growth, but as a growth inhibitor rather than accelerator.

The polymorphonuclear leucocytes have been shown by Opie (1922) to be much richer in tryptic leucoprotease than are the mononuclear leucocytes, and to be the chief source of leucoprotease. During acute inflammation they cause

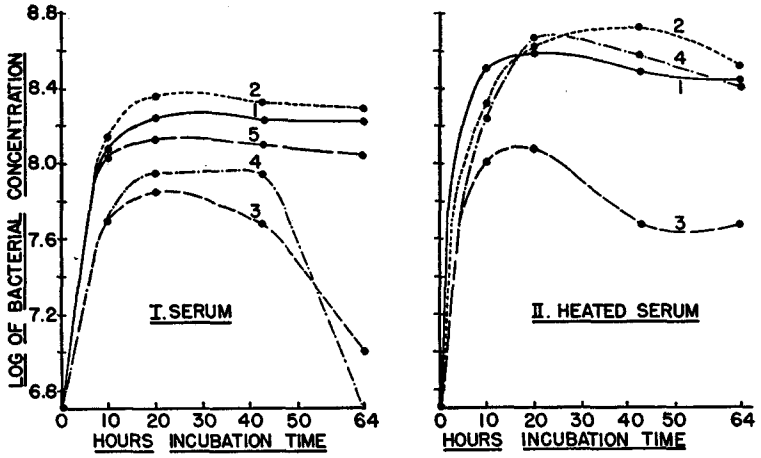

CHART A. Growth of *Escherichia coli*

a considerable accumulation of this enzyme. In diffuse phlegmonous inflammations and in serous cavities the leucoprotease is to a large extent buffered by the simultaneous exudation of fluid rich in serum antiprotease, while in abscess cavities and in cerebrospinal fluid such buffering action is minimal. In acute meningitis the increased meningeal permeability does allow some antiprotease to enter the cerebrospinal fluid, but this does not compare with the far greater amount of leucoprotease that is derived from the polymorphonuclear cells (Kaplan, *et al.*, 1939). That the uninhibited growth-accelerating action of the leucoprotease is at least in part responsible for difficulty of the body, even when aided by chemotherapeutic agents, to control the growth of organisms in acute meningitis, must be considered. Subdural injection of serum unfortunately results in only a temporary increase in the antiproteolytic activity of the spinal fluid, lasting not even 24 hours (Dochez, 1909).

In abscess cavities, too, a great preponderance of leucoprotease over anti-

protease occurs, since pressure within the confined space prevents the accumulation of fluid exudate, such as occurs in serous cavities and in phlegmonous inflammations (Opie, 1906). However, here, unlike the cerebrospinal spaces,

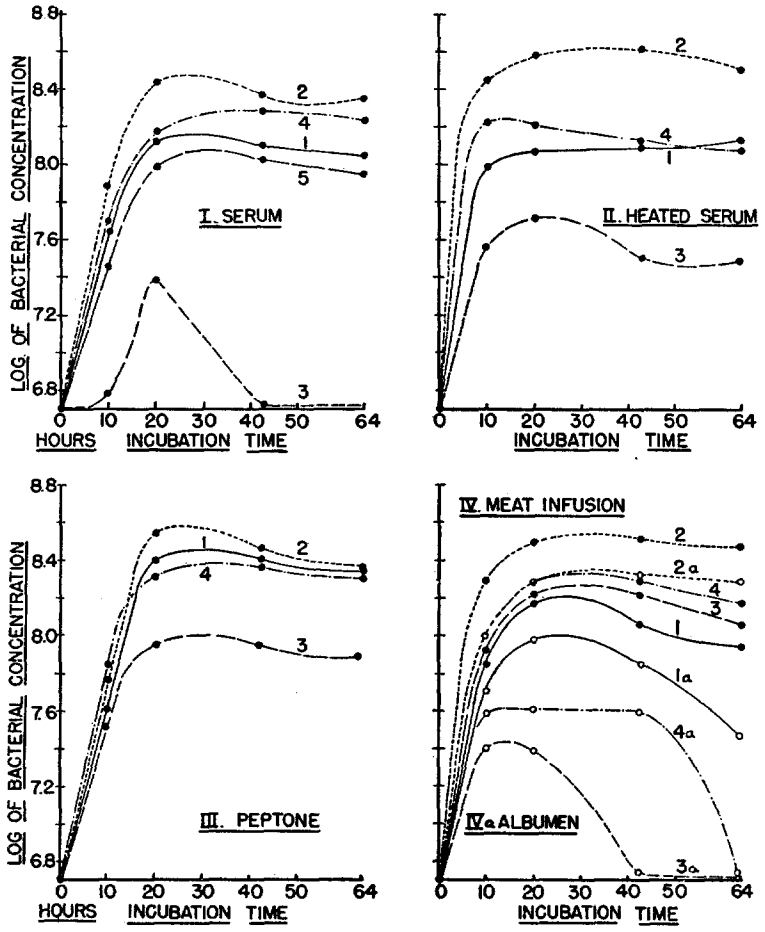

CHART B. Growth of *Eberthella typhi*

mechanical factors act to prevent spread of bacteria, and to inhibit their growth by causing a dearth of assimilable food and oxygen, and the accumulation of products of metabolism, including hydrogen ions. The preponderance of protease explains the extensive autolysis that occurs in abscess cavities, but that trypsin will penetrate and digest dead cells only (and probably only dead bacteria, too), and that living cells are not permeable to the enzyme, has been convincingly demonstrated by Northrop (1939). Hence protease would

not be expected to exert any bactericidal action by virtue of its enzymatic activity, although the accumulation of acid products of hydrolysis would, in an unbuffered medium, have both bactericidal and bacteriostatic activity as a

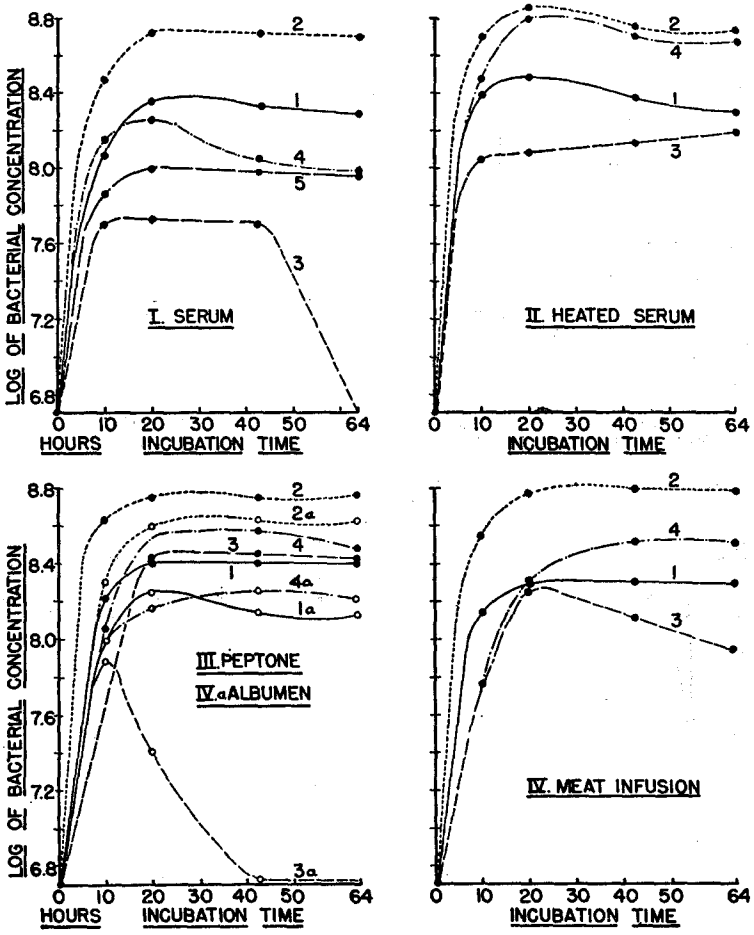

CHART C. Growth of *Proteus vulgaris*

result of increase in the concentration of hydrogen ions. The autolytic activity of protease (on dead organisms) is not evident in the experiments described above because the growth-promoting influences predominated during the time charted. After longer incubation more rapid decline in turbidity and loss of gram positive staining characteristics occurred in the presence of protease.

3. *In a limited number of experiments crystalline pancreatic trypsin inhibitor*

somewhat inhibited the growth of all the bacteria studied except Staphylococcus aureus. This finding supports the contention that antiprotease can be bacteriostatic, especially in media poor in non-protein nitrogen, and probably as

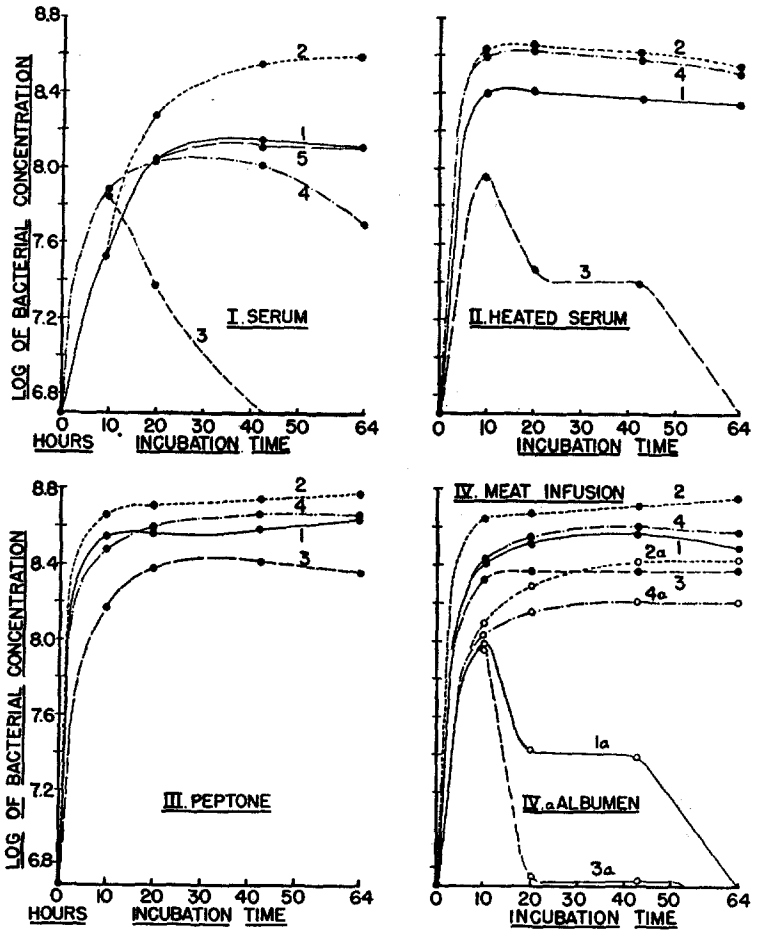

CHART D. Growth of *Staphylococcus aureus*

a result of inhibition of bacterial protease. Unfortunately insufficient crystalline inhibitor was prepared to study its action in other media than serum.

That trypsin inhibitor has no bactericidal activity, and trypsin no antibactericidal action, was demonstrated (for *E. typhi*) by incubating organisms (in peptone and in bactericidal serum) with trypsin inhibitor and with trypsin for 2 hours and testing successive dilutions for sterility after the method of Mackie and Finkelstein (1931).

4. *The bacteriostatic action of sulfathiazole was:*

(a) reduced by destruction of the antiproteolytic activity of the serum (by heat), (b) considerably reduced and sometimes almost obliterated by the presence of trypsin

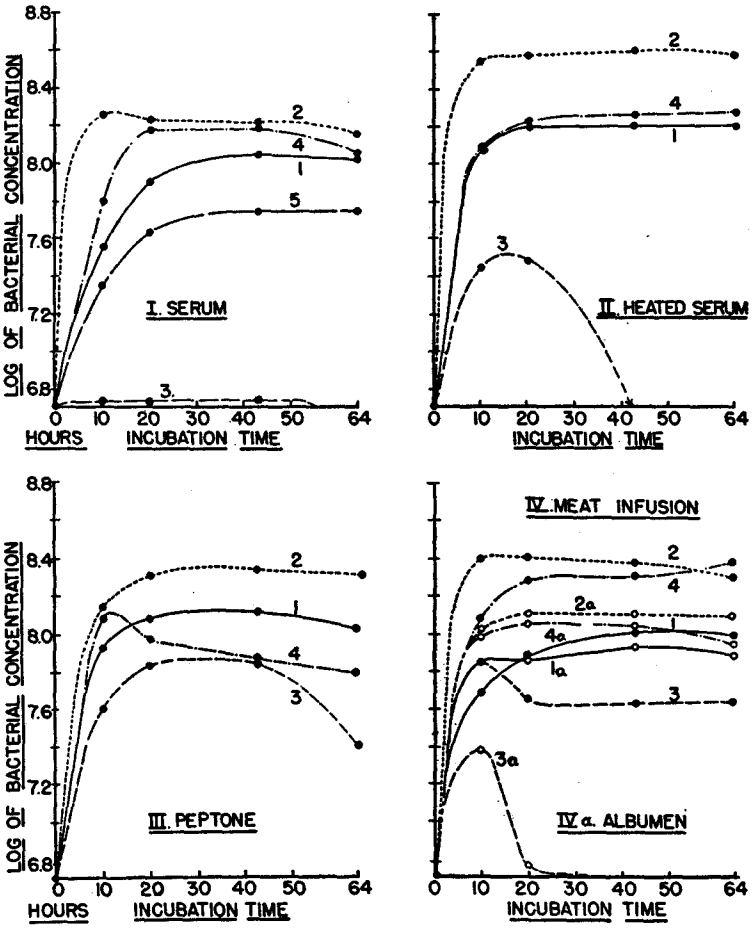

CHART E. Growth of *Streptococcus pyogenes*

in the medium, and *(c) greater in serum and albumen than in peptone and meat infusion.*

Since bacterial growth has been shown to be increased by destruction of serum antiprotease by heat, and by the addition of trypsin, the bacteriostatic action of sulfathiazole in these media would be expected to be correspondingly reduced, as compared to that in unaltered media. The greater bacteriostatic action in serum and albumen than in peptone and meat infusion indicates that

the products of protein hydrolysis not only promote bacterial growth, but also inhibit the bacteriostatic action of sulfathiazole.

The inhibitory effect of peptone and of various bacterial extracts on sulfanilamide has been known, and has been the subject of intensive study, for the past 4 years (Lockwood, 1938; McIntosh and Whitby, 1939; Stamp, 1939, etc.). Woods (1940) has offered evidence that *p*-aminobenzoic acid is essential for the growth of bacteria, that the enzyme reaction involved in the utilization of this substance is subject to competitive inhibition by sulfanilamide (due to structural relationship between the drug and the "essential metabolite"), and that the inhibitory effect of peptone and of various bacterial extracts is due to their content of *p*-aminobenzoic acid. He and his followers believe that the presence of *p*-aminobenzoic acid preformed in the medium is responsible for the difficulty of inhibiting bacteria by the use of sulfanilamide in peptone and in lesions characterized by considerable tissue destruction. They also believe that the sensitivity of organisms to sulfanilamide action depends on their inability to synthesize *p*-aminobenzoic acid. Recently, Bliss and Long (1941) have produced evidence that another protein breakdown product, methionine, possesses similar antisulfonamide action.

The experimental results recorded above indicate that the products of protein digestion, in addition to inhibiting the bacteriostatic activity of sulfathiazole, also directly promote bacterial growth. That the latter occurs in the presence as well as in the absence of sulfathiazole seems likely. The results indicate the importance with respect to bacterial growth and sulfonamide action of proteolytic enzyme (and peptidase?) in the medium, and therefore the importance of the buffering action of serum antiprotease on this enzyme.

If these conclusions are valid it is to be expected that the growth of bacteria in the body will be more rapid, and the inhibition of sulfathiazole will be greater:

(1) when considerable leucoprotease (and peptidase?) is present, (2) when the organism itself produces active protease (and peptidase?); (3) when the inflammatory exudate is small and poor in antiprotease; (4) when the source of antiprotease (the blood) is poor in this constituent; and (5) when the medium is rich in non-protein nitrogen (especially *p*-aminobenzoic acid?).

SUMMARY

1. Heating diluted serum at 80° C. for 10 minutes made it a better medium for bacterial growth. This is believed to have been at least partly due to destruction of the serum antiprotease.

2. Growth was accelerated, and proceeded further, in the presence of trypsin.

3. Growth was somewhat retarded in the presence of pancreatic trypsin inhibitor.

4. The bacteriostatic action of sulfathiazole in serum was reduced by heating the serum at 80° C., and much more markedly (in any of the media studied) by adding trypsin. It was greater in serum and albumen than in peptone and meat infusion.

5. The significance of the experimental results was considered in relation to the possible influence of leucoprotease and of serum antiprotease on bacterial growth and sulfonamide action.

Other Physiological Significance of the Antiproteolytic Activity of the Serum

Holmes, Keefer, and Myers (1935) have presented convincing evidence that the presence of antiproteolytic substances in synovial exudates is of great importance in the prevention of damage to joints by protease liberated from the leucocytes which accumulate in suppurative arthritis.

The possible importance of serum antiprotease in the prolongation of insulin action has recently been considered by Horwitt and others. That it may be of significance in this respect is rendered more likely by the demonstration and crystallization from the serum (Schmitz, 1937 and 1938) of small amounts of both protease and antiprotease.

In a series of papers (1935, 1936, 1937) Shute has reported experimental and clinical data designed to show that estrin possesses a special antiproteolytic activity (unlike that of serum antiprotease) which causes, when estrin is present in the blood in excess, abortion, premature labor, and abruptio placentae. He has also attempted to prove that the blood concentrations of estrin and of vitamin E are inversely proportional to each other, and that excess of estrin is usually due to vitamin E deficiency. His work has not yet been confirmed.

Rich and Duff have reported (1937) the experimental production of hyaline arteriolosclerosis and arteriolonecrosis within 24 hours after the injection of various proteolytic enzymes subcutaneously. Data presented under discussion of the nature and experimental variation of serum antiprotease indicate an inverse relation between the antiproteolytic activity of the serum and the local damage produced by trypsin. The relation (if any) of either of these factors to clinical arteriolosclerosis or arteriolonecrosis is not yet known.

The relation of serum antiprotease to the resolution and absorbtion of inflammatory exudates and absorbable foreign bodies (including catgut), and to organizing pneumonia, has been very little considered.

The use of serum (especially of high antiproteolytic activity) to neutralize the destructive protease liberated in acute pancreatitis and from duodenal fistulae has not yet been investigated.

The experiments of Menkin (1938), and of others, indicate that the increased capillary permeability and leucocyte infiltration characteristic of inflammation are due to a polypeptide formed by proteolysis at the site of inflammation.

The possibility of an inhibitory effect of serum antiprotease on this important proteolysis would seem to deserve investigation.

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